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Mamm. biol. **66** (2001) 84–89
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http://www.urbanfischer.de/journals/mammbiol

Mammalian Biology
Zeitschrift für Säugetierkunde

Original investigation

Biochemical identification of three sympatric *Apodemus* species by protein electrophoresis of blood samples

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Receipt of Ms. 28. 02. 2000
Acceptance of Ms. 20. 09. 2000

Abstract

The allelic pattern of seralbumine and general protein 1 of the three sympatric *Apodemus* species *Apodemus sylvaticus*, *A. flavicollis*, and *A. alpicola*, were studied using electrophoretic analysis of blood samples. This method appears to be a sensitive tool for distinguishing the three *Apodemus* species in the Alps. Their identification on the basis of external characteristics in the field is sometimes extremely difficult, even more so for juvenile specimens. Compared to previously described methods the electrophoretic analysis does not require killing animals and can be used on juveniles.

Key words: *Apodemus*, rodents, protein electrophoresis, biochemical identification

Introduction

The determination of the two common mouse species in western Europe, *Apodemus sylvaticus* (Linnaeus, 1758), and *A. flavicollis* (Melchior, 1834), is not always easy because their morphological characters strongly overlap. Some individuals or populations suggested hybridisation or introgression (ENGEL et al. 1973), but allozymatic studies did not reveal any hybrids (ENGEL et al. 1973; DEBROT and MERMOD 1977; NIETHAMMER and KRAPP 1978; GEMMEKE 1980; BENMEHDI et al. 1980; NASCETTI et al. 1980; CSAIKL et al. 1980; GEMMEKE and NIETHAMMER 1981; FRAGUEDAKIS-TSOLIS et al. 1983; NASCETTI and FILIPPUCCI 1984; GEBCZYNSKI et al. 1986).
Since the recognition of a third sympatric species, *A. alpicola* Heinrich, 1952, by STORCH and LÜTT (1989) with intermediate morphological traits, the discrimination became even more problematic. A better accuracy of species identification was obtained by a discriminant function developed from a limited number of skull measurements (REUTTER et al. 1999). Six cranial characters are sufficient to differentiate between the three *Apodemus* species with a correct classification above 97%. While, this technique is indeed a good tool for reclassifying museum material, it does not overcome the determination problem of young animals and of living individuals during field studies.
A discrimination independent of morphology should be based on genetic markers, e. g. specific allozyme pattern. The analysis

1616-5047/01/66/02-084 $ 15.00/0.

of allozyme variation by starch gel electrophoresis has frequently been used in genetic and systematic investigations of the genus *Apodemus* (ENGEL et al. 1973; BEHNMEHDI et al. 1980; CSAIKL et al. 1980; GEMMEKE 1980; FRAGUEDAKIS-TSOLIS et al. 1983; GEMMEKE 1983; KORPIMÄKI and NORRDAHL 1987; FILIPPUCCI et al. 1989; FERNANDES et al. 1991; BRITTON-DAVIDIAN et al. 1991; VAPA et al. 1995). VOGEL et al. (1991) and FILIPPUCCI (1992, 1996) included *A. alpicola* in their allozyme analysis and confirmed the specific status of this species. DEBROT and MERMOD (1977) found that the seralbumine pattern obtained by polyacrylamide gel electrophoresis is very distinctive for *A. sylvaticus* and *A. flavicollis.*

The aim of this study is to develop a technique applicable to all age cohorts of the three sympatric *Apodemus* species, *A. sylvaticus*, *A. flavicollis*, and *A. alpicola*, based on blood samples without need to sacrifice animals, in analogy with the techniques used for sibling species of shrews (HAUSSER and ZUBER 1983; BRÜNNER 1988; NEET 1989; NEET and HAUSSER 1989, 1990, 1991; BRÜNNER and NEET 1991; TURNI and SCHÖNHERR 1994).

Material and methods

Electrophoretic analysis was carried out on 41 individuals of the three species *Apodemus sylvaticus* (n = 15), *A. flavicollis* (n = 18), and *A. alpicola* (n = 8) from 11 localities in Switzerland and neighbouring Italy. Localities and collection numbers (IZEA: Institut de Zoologie et Ecologie Animale) of animals investigated are presented in the following list:

Apodemus sylvaticus Linnaeus, 1758. Switzerland: Bern: Haslital: (IZEA, 7381, 7382, 7383); Valais: Ayer: (7379); Monnaz: (4887, 4888); Vaud: Aclens (7380); Echichens: (4880, 4884); Morges: (4886); Renens: (7363, 7366); St. Saphorin: (4883, 4885). Italy: Domodossola: (7384).

Apodemus flavicollis Melchior, 1834. Switzerland: Bern: Haslital: (7389, 7390, 7392, 7393); Valais: Monnaz: (4894, 4895); Vaud: Aclens (7401); Le Brassus: (7395, 7396, 7397, 7398); Echichens: (4890); Morges: (4891); Renens: (7364); St. Saphorin: (4881, 4882, 4889, 4892).

Apodemus alpicola Heinrich, 1952. Switzerland: Valais: Sanetsch: (7337, 7338, 7339, 7345, 7346, 7347, 7348, 7361).

From every individual a blood sample of about 2 µl was taken from the base of the tail of the animals with heparinized Micro-Hematocrit tubes. The incision of the caudal vein with a razor blade (or another sharp blade) to get blood samples (maximum 0.1 ml) is recommended for mice by the Swiss Federal Office of Veterinary. By being transferred into an Eppendorf tube, the blood could be kept for more than 10 hours at ambient temperature (20 °C) or a week at a cool place (4 °C), and at least one year and even longer at –20 °C. This circumstance allows the use of animals for the electorphoretic analysis that were frozen a few hours after death.

Blood samples were diluted in a solution (1:5) of saccharose (40%) and 0.075 M Tris/HCl buffer, pH 8.9 with a trace of bromophenol blue. The added amount of the saccharose-buffer solution depended on the volume of the blood sample. For example, to a 2 µl blood sample 8 µl saccharose and 50 µl buffer solution were added. The samples were then run in a Polyacrylamid-disc-electrophoresis (resolution gel: 8%, 0.325 M Tris/HCl, pH 8.9; concentration gel: 3%, 0.056 M Tris/HCl, pH 6.9; running buffer: 0.05 M Tris/HCl, 0.38 M glycerine) with a constant power of 4 W during migration in the concentration gel and 12 W in the resolution gel (gel size: 180 × 155 × 15 mm; power supplies: Bio Rad 3000/300 and 1000/500; gel support: Zabona AG, Basel). Proteins migrated from cathode to anode during 4–5 h (band of bromophenol blue at 1 cm from lower gel border). Proteins were non-differentially stained with Coomassie blue (0.025% Coomassie blue R250, Sigma; 50% methanol; 3.5% glacial acetic acid) for 1 h and destained afterwards (50% methanol; 3.5% glacial acetic acid). The method is slightly modified according to HAUSSER and ZUBER (1983).

Relative migrating distances of the proteins in relation to the bromophenol blue dye front were calculated to identify clearly the different protein bands.

All skulls of the examined specimens were prepared, measured, and assigned to one *Apodemus* species by using a discriminant function analysis (REUTTER et al. 1999).

To test our results we included 58 unknown *Apodemus* specimens from the Bündner Natur-Museum Chur. All these animals came from the eastern part of the Swiss Alps (Graubünden). Blood samples were taken with heparinized Micro-Hematocrit tubes from the defrosted bodies.

Results

The results of the electrophoretic analysis (Fig. 1) are best understood when considering the different allelic pattern of the albumin and an unknown general protein 1 (GP 1). These two proteins are represented on the gel by three different bands which are labelled from cathode to anode as A, B, and C. *Apodemus flavicollis* and *A. alpicola* are characterised by the slower migrating albumin allele (band A), whereas the faster migrating albumin allele (band B) is present only in *A. sylvaticus*. Moreover, *A. flavicollis* shows an additional protein band C (GP 1), which migrated further than either A and B. Nothing is known about the identity of GP 1, its possible polymorphism and the position of other allelic bands in *A. alpicola* and *A. sylvaticus*. However, the presence of the characteristic GP 1 band in *A. flavicollis* allows the distinction between this species and *A. alpicola*.

When the migration distance of band A is taken as 100%, the relative distances are for band B 101.6% (101.3–102.0) and for band C 107% (105.2–108.5) (Tab. 1). In that way 100% of all examined specimens could be determined unequivocally.

All 58 test specimens from Graubünden could be identified according to the protein electrophoresis of blood samples. 52 individuals were assigned to *A. sylvaticus*, five to *A. flavicollis* and only one to *A. alpicola*. Skull measurements using a discriminant function (Reutter et al. 1999) confirmed this determination.

Table 1. Protein markers and bands (Alb and GP 1) of the three species *A. sylvaticus* (B), *A. flavicollis* (A, C) and *A. Alpicola* (A).

marker	band	*A. sylvaticus*	*A. flavicollis*	*A. alpicola*
Alb_{100}	A		x	x
$Alb_{101.6}$	B	x		
GP 1_{107}	C		x	

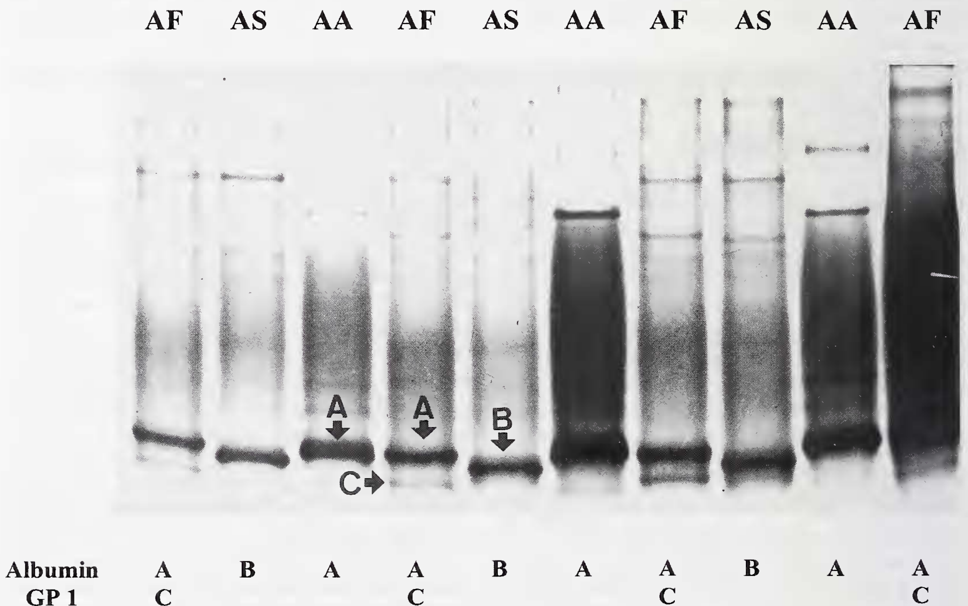

Fig. 1. The serum albumin and general protein (GP 1) patterns obtained by protein electrophoresis on polyacrylamide gels for *A. sylvaticus* (B), *A. flavicollis* (A, C) and *A. alpicola* (A). Symbols: AF = *A. flavicollis*, AS = *A. sylvaticus*, AA = *A. alpicola*.

Discussion

The determination of the three morphologically similar species *A. sylvaticus*, *A. flavicollis*, and *A. alpicola* remains sometimes difficult. *A. sylvaticus* and *A. flavicollis*, are easily distinguishable in northern Europe by morphological characteristics and by the ecological parameters of their habitats. *A. flavicollis* is larger, with a complete collar of yellow-reddish or wide spot on the breast, and inhabits forest. *A. sylvaticus* is smaller and an eurytopic species with an elongated pectoral spot never forming a collar or without any spot at all. These two species converge morphologically in southern Europe, due to clinal variation in body size and pelage colour following opposite trends (*Engel* et al. 1973).

Hence, convergence and overlapping in external characters do not always allow a correct specific assignment of specimens, especially in areas where the two sibling species are distributed sympatrically, and when juvenile individuals are concerned. The recognition of *A. alpicola* further complicated this determination problem in certain regions. The alpine mouse resembles the wood mouse in pelage colour while in body size it resembles the yellow-necked mouse.

The present results clearly show that the three species *A. sylvaticus*, *A. flavicollis*, and *A. alpicola* can be 100% distinguished biochemically by their albumin and general protein 1 (GP 1) patterns. The electrophoretic patterns of the albumin and the GP 1 of *A. sylvaticus* and *A. flavicollis* in the present study correspond to those of Debrot and Mermod (1977), who also analysed animals from Switzerland using the same technique. Darviche et al. (1979) reported two specific albumin alleles for *A. sylvaticus* and *A. flavicollis* from France, Corsica, Spain, and Italy, and suggested that these differences are good criteria for differentiation between the two species. Moreover, it has been shown that *A. alpicola* has an intermediate position between *A. sylvaticus* and *A. flavicollis* with regard to allozyme allele frequencies (Vogel et al. 1991; Filippucci 1992). For the albumin locus, the species *A. alpicola* and *A. flavicollis* share the same allele.

In all analysed individuals of the present study (including the test animals from Graubünden) no heterozygotes were found. These findings support the hypotheses that there is no gene flow between these taxa.

The fact that the two alleles do not show a very pronounced difference in gel migration may lead to problems in the case of a monospecific sample of *A. sylvaticus* and *A. alpicola* or a mixture between these species. Therefore, we recommend to load reference samples of the two more common species *A. sylvaticus* and *A. flavicollis* on every gel.

For ecological studies the electrophoretic analysis of blood samples offers not only the advantage that the animal need not be sacrificed as well as the identification of juvenile individuals, what was impossible with previously described methods. Moreover, the blood samples can be taken from living, freshly killed (even after several hours), as well as frozen animals and can be stored for months at –20 °C. The application of the technique is simple (duration of the whole laboratory procedure about 5 hours). All these advantages allow an application for ecological and long-term studies in the field.

Acknowledgements

We would like to thank Nelly Di Marco, who performed most of the electrophoreses. We are also very grateful to Jürg Paul Müller from the Bündner Natur-Museum Chur (Switzerland), and to Etienne Bertouille who provided us with a lot of wood mice and to Tiziano Maddalena and Manuel Ruedi, who gave us a lot of precious technical advices. Last, but not least, we thank Karen Parker who corrected the English manuscript.